NATIONAL INSTITUTE FOR BIOLOGICAL STANDARDS AND CONTROL

Division of Bacteriology

Standard Operating Procedure

In vitro SNAP-25 endopeptidase Immunoassay for potency testing of botulinum type A toxin preparations

Written by: Y Liu

Authorised by: D Sesardic

INTRODUCTION

The L-chain of Botulinum neurotoxin type-A is known to contain a zinc-dependent endopeptidase enzyme whose toxic action results from cleavage of the critical synaptic protein SNAP-25. The assay is based on the immunochemical estimation of the cleavage of the SNAP-25 substrate by BoNT/A. A fragment of SNAP-25 spanning the toxin cleavage site (137-206) is immobilized onto wells of a microtitre plate and subsequently treated with BoNT/A. This results in a new epitope (SNAP-$25_{190-197}$) being exposed which was previously concealed. This new epitope is then measured with a specific antibody (anti-SNAP-$25_{190-197}$) to obtain an estimate of toxin endopeptidase activity.

MATERIALS AND REAGENTS

Materials

NUNC Maxisorb ELISA plates
Graduated pipettes (25ml, 10ml, 5ml)
Multichannel pipette and tips
Gilson (P1000, P200, P100, P20) (in calibration)
Measuring cylinder (1L)
Multiscan ELISA plate reader running Genesis software
pH meter (in calibration)
Electronic balance (in calibration)

Reagents

Coating Buffer (0.1M $NaHCO_3/CO_3$, pH 9.6)
Phosphate Buffer Saline (PBS)
PBS + 0.05% Tween-20 (PBST)
Marvel (skimmed milk powder)
HEPES (Sigma, H-3375, FW: 238.3)
Bovine Serum Albumin (BSA, Sigma, A0281, fatty acid free, globulin free)
DL-Dithiothreitol (DTT, Sigma, D5545)
Synthetic peptide substrate SNAP-$25_{137-206}$

Toxin:
Botulinum Toxin A (in house product specific reference, product batch)

Pre-reduction buffer: 50mM HEPES-NaOH, pH 7.0, + 20µM $ZnCl_2$, + 10mM DTT

Reaction Buffer: 50mM HEPES-NaOH, pH 7.0, + 20µM $ZnCl_2$, + 5mM DTT + 1mg/ml BSA

Substrate: Synthetic SNAP-25 peptide (SNAP-$25_{137-206}$):

70aa substrate (GGFIRRVTND ARENEMDENL EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT KMLGSG) [Ekong *et al*, 1997]. Synthesized and purified (>80% pure).

Stock solution @10mg/ml aliquoted and stored at -20C.

Detecting antibody: Primary Antibody: Site-specific anti-peptide (CTRIDEANQ) antibody raised in New Zealand White rabbits. Specificity as reported in Ekong *et al*, 1997, Ekong *et al*, 1995.

Secondary Antibody: Goat anti-rabbit HRP conjugate (Sigma, A0545)

Substrate: 2, 2'-Azino-bis (3-ethylbenzthiozoline 6 sulfonic acid) (ABST) (Sigma, A9941)

Calibration checks and equipment checks related to this SOP

Before commencement of assay the following equipment calibrations must be carried out:

1. Calibrations of balance according to Bacteriology (NIBSC SOP QM005)
2. Calibrations of pH meter to pH 4 and 7
3. Calibrations of Gilson pipettes annually by qualified technician and a four monthly validation check laboratory personnel (NIBSC SOP PIP)

Unless otherwise stated in the SOP there is no requirement to use volumetric glassware in traceable calibration of the preparation of reagents, solutions or dilutions used in this SOP. Semi-automated pipettes, disposable plastic graduated pipettes, syringes, measuring cylinders and glassware are appropriate to the volumes being used, are adequate for this purpose. Volumes less than 1ml are dispensed using Gilson pipettes in calibration. All reagents used in the preparation of solutions should be General Purpose Reagent grade, unless otherwise stated.

PROCEDURE FOR ENDOPEPTIDASE ASSAY

Immobilisation of SNAP-25 substrate

1. Prepare solution of 2µg/ml synthetic SNAP-25 substrate in coating buffer.
2. Add 100µl/well of SNAP-25 substrate solution to 96-well plates
3. Incubate at 4^{o}C overnight.
4. Next day, wash plates 3x in PBST.
5. Add 150µl/well of 5% Marvel in PBST (M-PBST) to block.
6. Incubate for 1h at 37^{o}C in a humidified box
7. Wash plates 3x with dH_2O and blot dry for immediate use

N.B Sealed plates can be stored at -20^{o}C for up to 2 months.

Treatment of immobilised substrate with toxin

1. For toxin samples with 100U/vial: 8 vials of toxin reference, 6 vials of toxin test sample are needed
   For toxin samples with 500U/vial: 4 vials of toxin reference, 3 vials of toxin test sample are needed
   (NB. 2 Samples can be tested in 1 assay)

2. Label toxin sample vials as follows:
   S1, S2, S3
   T1, T2, T3 (if more than 1 sample is to be assay)
   Label product specific reference vials as R1, R2, R3, R4, A (plate control and main reference)

3. Reconstitute test sample(s) and product specific reference toxin sample in Pre-reduction buffer as follows:

Carefully remove caps and stoppers ensuring that none of the material is lost.

Sample A (100U/vial): Add 100µl/vial of Pre-reduction buffer.
Pool vials of the same preparation to give a total of 3 samples vials and 4 reference vials.

Sample B (500U/vial): Add 300µl/vial of Pre-reduction buffer

4. Gently mix to ensure all the material is dissolved and incubate vials for 20mins at 37°C.

5. Place SNAP-25 coated plates on ice. (if plates have been stored, wash plates 3x in dH_2O and blot dry prior to placing on ice)

6. Remove vials from incubator and place on ice.

7. Add 180µl/well of Reaction buffer to row A
   Add 100µl/well of Reaction buffer to the remaining wells (rows B-H)

8. Add 20µl reduced toxin per well to Row A according to the plate layouts show below.

   The plate layout is designed to minimize the edge effect and the main reference (A) is located in the centre of the plate to avoid any variations in the absorbance.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
PLATE 1	S1	S1	R1	R1	T1	T1	A	A	T2	T2	S2	S2
PLATE 2	T2	T2	S2	S2	R2	R2	A	A	T3	T3	S3	S3
PLATE 3	T3	T3	S3	S3	T1	T1	A	A	R3	R3	S1	S1
PLATE 4	T2	T2	S2	S2	R2	R2	A	A	R3	R3	S1	S1

9. Perform doubling dilutions down each plates straight after adding sample/reference to row A

10. Seal the plates individually with self-adhesive tape and incubate plates in a humidified container for 60mins at 37°C (do not stack).

Estimation of immobilized intact and cleaved SNAP-25 substrate

1. Wash toxin treated plates 3x in PBST. Blot dry.
2. Add 100µl/well of 5µg/ml R-14 in 2.5% M-PBST (Primary-Ab)
3. Incubate for 90mins at 37°C
4. Wash plates 3x in PBST. Blot dry.
5. Add 100µl/well of 1/2000 goat anti-rabbit-HRP conjugate in 2.5% M-PBST
6. Incubate for 90mins at 37°C
7. Add 100µl/well ABTS substrate solution
8. Allow colour to develop at room temperature (leave for ~30-45mins)
9. Shake and read absorbance at 405nm using the ELISA plate reader.

Statistical Analysis

1. Absorbance reading obtained from Multiscan plate reader is transferred to word document with the appropriate plate layout and dilutions.

2. Using the bioassay program RANDOM, three points are chosen that are liner and parallel to the plate control reference (reference). The potency of the test samples is calculated relative to the main reference (A) by multiply the potency value of the test sample (not the log form) by the assigned unit/vial value of the main reference. Thus expressing the potency relative to the reference.

3. The potency values of the test samples is entered into Excel work sheet to calculate the overall mean of the test samples (± S.D) [see example]

4. The mean potency value of the test samples obtained from the *in vitro* endopeptidase assay needs to fall within the Manufacturer's Specifications and limits (±15%) or the European Pharmacopoeia specifications and limits (±20%) in order to be released by NIBSC.

   If test sample fails to meet either specification the sample is assayed in vivo using the mouse local flaccid paralysis assay.

Example:

Layout

	1	2	3	4	5	6	7	8	9	10	11	12
Plate1	S1	S1	R1	R1	T1	T1	A	A	T2	T2	S2	S2
Plate2	T2	T2	S2	S2	R2	R2	A	A	T3	T3	S3	S3
Plate3	T3	T3	S3	S3	T1	T1	A	A	R3	R3	S1	S1
Plate4	T2	T2	S2	S2	R2	R2	A	A	R3	R3	S1	S1

Vial	**Replicate 1**	**Replicate 2**	**Replicate 3**	**mean**		**plate control (REF)**	
S1	711	598	532	613.7		650	
S2	732	499	433	554.7		605	
S3	502	515		508.5		597	
						537	
						593	
						596.4	**40.27**
		Overall mean (S.D.) of # Test Sample =		558.9	52.71		

BUFFERS FOR ELISA

A. Phosphate Buffered Saline pH.7.4 (20x)

CHEMICAL	AMOUNT
Sodium chloride	800g
Potassium dihydrogen orthophosphate	20g
Di-Na-H-orthophosphate (dihydrate)	143g
Potassium chloride	20g
Distilled Water	Made up to 5 Litres

B. PBS/0.05% Tween

CHEMICALS	VOLUME
20x PBS solution	250ml
Tween 20	2.5ml
Distilled Water	Made up to 5 Litres

C. 0.05M Citric Acid, pH 4.0 (ABTS Substrate buffer)

CHEMICALS	AMOUNT	AMOUNT (2.5L)
Citrate monohydrate	10.51g	26.28g
Distilled Water	Made up to 1 Litre	Made up to 2.5 Litres

D. Carbonate buffer, pH 9.6 (ELISA plate coating buffer)

CHEMICALS	VOLUME	VOLUME (2.5L)
Sodium carbonate, 0.015M	1.59g	3.98g
Sodium Hydrogen carbonate,0.035M	2.93g	7.33
Distilled Water	Made up to 1 Litre	Made up to 2.5 Litres

- Store at 4^{o}C for up to 2 weeks. For long term storage, autoclave (121^{o}C for 15mins), store up to at 4^{o}C

E. 50mM HEPES-NaOH buffer, pH 7.0 + 20µM $ZnCl_2$

CHEMICALS	AMOUNT	AMOUNT (2.5L)
50mM HEPES	2 litre	2.5 Litres
10mM $ZnCl_2$	4ml	5ml

- 50mM HEPES: 11.915g in 1 litre dH_2O
- 10mM $ZnCl_2$ (FW: 136.3): 0.0136g in 10ml dH_2O
- Dilute $ZnCl_2$ to 20µM in HEPES buffer (1/500 dil). Adjust pH to 7.0 using 10M NaOH and Store at 4^{o}C.